Post-transcriptional mechanisms in type XVII collagen synthesis

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CHAPTER 1

INTRODUCTION

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ABSTRACT

The main function of the protein type XVII collagen, formerly known as BP180 or BPAG2, is anchoring the epidermis to the underlying dermis. The function of type XVII collagen can be impaired due to either an auto-immune reaction against or genetic deficiency of the molecule, which then leads to the development of blistering diseases.
Research over the years has mainly focused on understanding this adhesive function and less attention was given to understanding its basic regulation of transcription and translation. As it appeared that the protein may exert a very dynamic expression level and is also heterogeneous at both the mRNA transcript and at the protein level, it seems to make sense to study its basic features in detail. Also nearing the chances that gene therapy, applied to repair defective *COL17A1* alleles, will be successfully requires a thorough understanding of which factors control expression of the protein and its derivatives[1]. At this moment, however, many aspects are largely not understood.
In this chapter we try to highlight the major observations involving most functional aspects of type XVII collagen and to indicate what aspects need further investigation. Moreover, we will focus on other supposed functions of type XVII collagen. At the end of this introduction, we will point out the most intriguing subjects in connection with the aim of this study.

Heterogeneity of type XVII collagen at the mRNA and the protein level

The human gene coding for type XVII collagen, *COL17A1*, is located on the long arm of chromosome 10, in band 10q24.3. It spans 52 kb of genomic DNA and contains 56 exons that finally lead to a coding sequence of 4491 nucleotides[2-5]. This number of exons is substantially higher than the mean number per gene (8.8) in the human genome[6].
The coding sequence is highly conserved between species; *Mus musculus* (mouse) and *Homo sapiens* coding sequences showing a 86% overlap[4,7]. Other known (partial) *COL17A1* sequences include *Canis familiaris* (dog), *Mesocricetus auratus* (hamster), *Sus scrofa* (pig), and *Gallus Gallus* (chicken)[8-11].

By use of bullous pemphigoid and herpes gestationes patient sera the first cDNA clones that contained *COL17A1* sequences were obtained in 1990[2]. Within two years this led to the chromosomal assignment of the gene and the discovery of the complete ORF sequence, which showed on Northern blot as a 6 kb transcript[4,7]. For eight years it was assumed that this was the only transcript until in 2000 Molnar *et al* showed that alternative splicing generated two messengers that differed 0.6 kb in size[12].
This alternative splicing leads to two mRNA variants that have the same ORF but differ in their 3'UTR. One variant, hereafter referred to as the long variant, contains the full-length 3'UTR, whereas the second variant, hereafter called the short variant, lacks 610 nucleotides within the 3'UTR (Fig. 1). The long variant is the major transcript in normal human keratinocytes, whereas the short variant predominates in the squamous carcinoma cell line UMSCC-22B. In the long variant 3'UTR four stretches are found which are highly conserved (78-87%) between the human and the mouse sequence. The homology here is significantly higher than the mean homology found between 3'UTRs of human and mouse[13].
The function of this differential splicing is still completely unknown, but could involve functions that nowadays are attributed to 3'UTRs such as control of translation level, transcript stability, and subcellular mRNA localisation.
Extensive sequencing of ORF amplimers in many laboratories, partly as search strategies for *COL17A1* mutations in type XVII collagen deficient patients, has not unveiled any discrepancies at the coding sequence level. The sequence of the 5'UTR, however, is still elusive. The *COL17A1* 5'UTR of only one species has so far been found; the murine 5'UTR spans 306 nucleotides and the sequence is highly homologous to the human DNA sequence upstream of the first exon[7].

The protein was originally identified as a 180 kDa antigen that could be visualized by pemphigoid patient sera in Western blotting on epidermal cell extracts. Deduction

from the ORF sequence gives 155 kDa of amino acid sequence. It furthermore contains several kilodaltons of extracellular carbohydrate moieties and can be phosphorylated[14,15]. It has a type II transmembrane orientation, thus with a COOH-terminal ectodomain and an NH_2-terminal cytoplasmic domain. The ectodomain comprises two-third of the entire molecule and contains 15 collagenous subdomains (COL1-COL15) by which the molecular tail folds into a triple-helical structure. As a result the final shape of the trimer is a globular head with a central rod and a flexible tail (Fig. 1)[4,16,17].

Immunoelectron microscopy against two different epitopes in the ectodomain demonstrated that the ultrastructural localisation of these epitopes differed that way that it must be assumed that the flexible tail forms a loop in the lamina lucida, thus with the COOH-terminal end lying higher in the lamina lucida than the mid-portion of the tail[16].

This triple helix folding supposedly occurs in an N- to C-terminal way, which is the opposite of the classic fibril-forming of pro-collagens[18]. In this the folding process the three monomer chains may interact by a specific nucleation site in the NC16A subdomain, that precedes the fifteen collagen sub-domains, to allow for the formation of a proper stagger in the COL15 domain after which the folding can protrude in the C-terminal direction.

The main function of the cytoplasmic domain is to present a binding platform in the intracellular part of the hemidesmosome for other adhesion molecules, that will be discussed in more detail below.

The second form of type XVII collagen is the soluble 120 kDa ectodomain also called LAD-1 (Fig. 1)[14,19]. Originally the molecule was discovered in 1996 by Marinkovich *et al* when they immunoblotted sera of patients having linear IgA dermatosis[20]. Although first thought to represent a separate antigen, one year later Pas *et al* demonstrated that bullous pemphigoid and linear IgA dermatosis sera recognized a same 120 kDa antigen, and that this antigen was very similar to the ectodomain of type XVII collagen[14]. The form is also present as a separate homo-trimer and thus it does not form mixed trimers with the full-length type XVII collagen molecule[21].

The cleavage product was first demonstrated in the medium of cultured keratinocytes and antibody mapping showed that it only contained epitopes also present in the ectodomain of the full-length molecule. A polyclonal antibody against NC16A revealed that most of this domain must present in this cleaved form. Rotary-shadowed images showed that this molecule is composed of the central rod and the flexible tail, the globular head of the founder molecule is, as expected, lacking[19]. Pas *et al* suggested that the soluble form was generated either by alternative splicing or by proteolytic cleavage[14]. Five years later it was showed that the soluble form is probably shed from keratinocytes by metalloproteases, in particular ADAMs, a family of sheddases. Three members of the ADAM family, TACE, ADAM-9, and ADAM-10, can shed type XVII collagen in vitro, although TACE-deficient keratinocytes still showed a residual shedding of 60%. Moreover, silencing the gene

of the major sheddase, TACE, with siRNAs resulted only in reducing type XVII collagen cleavage to half of its original level and the actual contribution of ADAM-9 and ADAM-10 to the shedding of the ectodomain has not been investigated[22-24]. This suggests that the cleavage of type XVII collagen to its 120-kDa form may involve sheddases, but that other unidentified factors may also be necessary.
By deleting specific sequences within the NC16A domain it was defined that the cleavage site ranged from amino acid 528 to 547. Software prediction of secondary structures of these deletion mutants revealed that non-shed mutants formed a new amphipatic α-helix, which disturbed the tertiary structure of the homo-trimeric type XVII collagen molecule. One large deletion mutant showed no changes in secondary structure with respect to the original entire NC16A sequence and the non-cleavage here was explained by the residual NC16A being to short to allow cleavage[24].
The supposed shedding of the LAD-1 molecule may be accompanied by modifications to the amino acid sequence as autoantibodies in linear IgA disease preferentially target LAD-1 rather than the full-length protein. Moreover, sera of bullous pemphigoid patients, that in general recognise BP180 and/or BP230 may sometimes only target the LAD-1 molecule[14,25,26]. This suggests that the soluble ectodomain contains neo-epitopes that apparently are also immunogenic.

Besides LAD-1 a second soluble form has been described, LABD97, that is somewhat smaller, having a molecular weight of 97 kDa (Fig. 1). Originally it was described by Zone *et al* as an antigen in linear IgA dermatosis[27]. The 97 kDa form supposedly is a product of C-terminal cleavage of LAD-1, since both forms contain the NC16A domain[19,20]. Antibodies raised to the 97 kDa form locate it in the lamina lucida closely spaced to the ectodomain of type XVII collagen, and it has been suggested that it may form a complex with this molecule[28].
The two soluble forms of type XVII collagen have different N-termini; the N-terminus of LAD-1 appears to be seven amino acids upstream compared to that of LABD97 (Alanine-531 versus Leucine-524)[29,30]. The reason for this difference remains unclear. As mentioned above, the 97-kDa antigen may be proteolytically produced by cleavage of the C-terminal part of the 120-kDa antigen. It is conceivable that the same proteases cleave LAD-1 at the N-terminus. A hypothesis to explain the N-terminal difference may be that ADAMs determine their cleavage site on basis of fixed distance to the transmembrane domain rather than a specific recognition site. Slight differences in the molecular conformation of type XVII collagen in in vivo skin may influence the cleavage site. Another hypothesis is that the 97-kDa antigen is a product of distinctive cleavage processes[29]. The latter hypothesis seems the most favourable one.

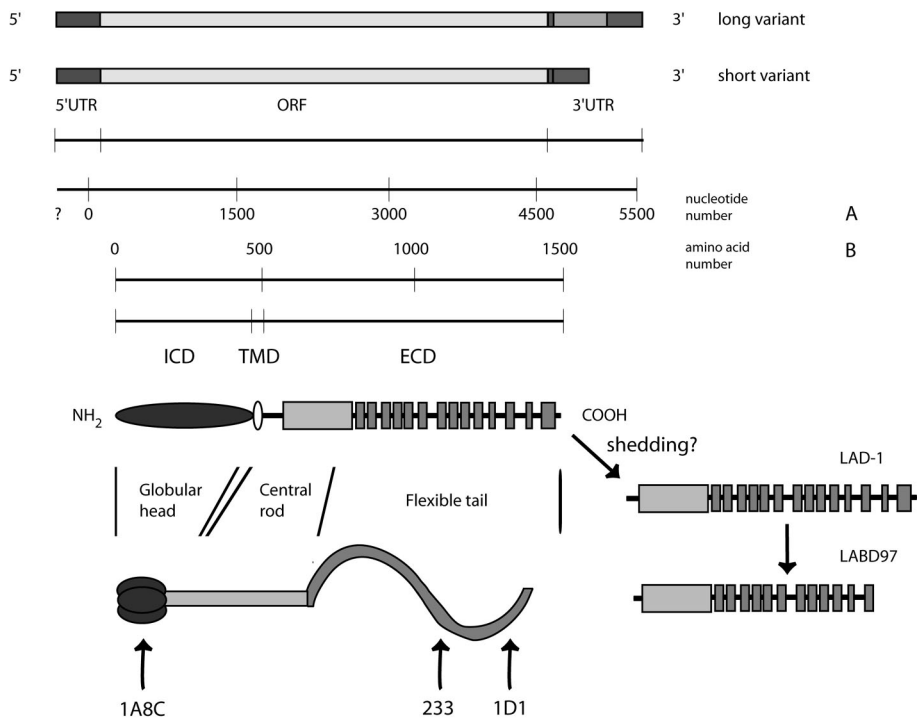

Figure 1. Heterogeneity of type XVII collagen at the mRNA and protein level.
Schematic depiction of both mRNA and protein structures of type XVII collagen. (**A**) *COL17A1* mRNA consist of two mRNA variants, one containing the full-length 3'UTR (in light and dark grey), hereafter referred to as the longer, whereas the second variant, hereafter called the short variant lacks 610 nucleotides within the 3'UTR (in dark grey). Moreover, the exact sequence of the 5'UTR is still unknown. (**B**) Type XVII collagen has a type II transmembrane orientation, thus with a COOH-terminal ectodomain and an NH_2-terminal cytoplasmic domain. The ectodomain comprises two-third of the entire molecule and contains 15 collagenous sub-domains (COL1-COL15: depicted in dark grey) by which the molecular tail folds into a triple-helical structure. Folding is initiated at the NC16A domain, after which a proper stagger is formed in the COL15 domain (in light grey). The folding can then protrude in the C-terminal direction. As a result the trimer takes on its final shape of a globular head with a central rod and a flexible tail. Epitopes detected by monoclonal antibodies 1A8C, 1D1, and 233 are depicted with arrows. All three monoclonal antibodies are suitable for immunohistochemistry, immunofluorescence and Western blot. The ectodomain can be shed of the cell membrane, possibly through proteolytic cleavage, resulting in a secreted form of 120 kDa (LAD-1), which can then be further cleaved to a second soluble form of 97 kDa (LABD97). UTR: untranslated region, ORF: open reading frame, ICD: intracellular domain, TMD: transmembrane domain, ECD: extracellular domain. Partly adapted from Hirako *et al*[17], for further references see text.

Whether the soluble forms have a functional meaning is still unknown although some suggestions have been done in the literature. Cleavage of the ectodomain may influence keratinocyte motility, detachment, and differentiation. Transfection of HaCaT keratinocytes with cDNAs of involved sheddases, TACE, ADAM-9, and ADAM-10, did not only enhance cleavage of the ectodomain but also altered cell motility. This evidence is, however, not very convincing since no significant differences in cell motility with control HaCaT keratinocytes were observed. Detachment may also influenced by shedding since it can be imagined that keratinocytes have lost binding partners after shedding[22]. In addition, Hirako *et al* suggested that shedding by metalloproteases contribute to hemidesmosomal turnover[23]. This would imply that the soluble forms are no more than garbage left behind by basal cells that have migrated to the upper layer. Others have suggested that cleavage of the ectodomain may render keratinocytes unresponsive for possible ligands of type XVII collagen as the soluble ectodomain may then modulate the activity of those possible ligands[31]. Moreover, the emerging N-terminus of the ectodomain may also contact other ligands on keratinocytes.
The only sound conclusion at this moment is that more detailed cell biological analyses are necessary to determine the role of shedding.

Location and function of type XVII collagen

The main function of type XVII collagen is the anchoring of the basal cells of the epidermis to the underlying basement membrane. Its adhesive function is exerted in combination with several other proteins that together form the hemidesmosome, to be precise the type I hemidesmosome (Fig. 2). The importance of type XVII collagen in this complex is emphasised by the severe blistering of the skin when the function of type XVII collagen is impaired by mutations in the *COL17A1* gene[32,33]. The protein may also be involved in motility of keratinocytes, since type XVII collagen deficient keratinocytes show increased movement compared to normal keratinocytes[34].
Furthermore, incubation of healthy human keratinocytes with IgG autoantibodies from sera of patients with pemphigoid diseases result in increased secretion of two specific cytokines, IL-6 and IL-8, whereas secretion of other cytokines is not altered. Pre-adsorption of these autoantibodies with recombinant stretches of the NC16A domain abolishes this secretion. These observations indicate a role for type XVII collagen in signal transduction in which the precise pathway still has to be resolved[35].
Type XVII collagen can be phosphorylated by protein kinase C (PKC) and this may cause disassembly of hemidesmosomes. When type XVII collagen was phosporylated by use of 12-O-tetradecanoylphorbol-13-acetate (TPA), the protein acquired a higher apparent molecular weight on SDS-PAGE, which could be abolished by a selective PKC inhibitor. Treatment of cells with TPA also changed the localisation of type XVII collagen from the cell surface to a smaller ring pattern in the cytoplasm[36,37]. Disassembly of hemidesmosome by phosphorylation of

another component of the hemidesmosome, integrin α6β4, has already been investigated and will be discussed in more detail below.

Hemidesmosomal structure

Hemidesmosomes are adhesion structures that provide a strong interaction between basal cells and the underlying basement membrane. They consist of at least six different proteins: the type XVII collagen, the plakin protein BP230, the α_6 and β_4 subunits of integrin $\alpha_6\beta_4$, tetraspanin/CD151 and plectin (Fig. 2)[4,38-44].
Hemidesmosomes form the bridge between cytoskeleton and the extracellular matrix components. Hemidesmosomes appear in two different types: type I hemidesmomes contain all components, whereas type II hemidesmosomes only consist of integrin $\alpha_6\beta_4$, tetraspanin, and plectin[45-47].

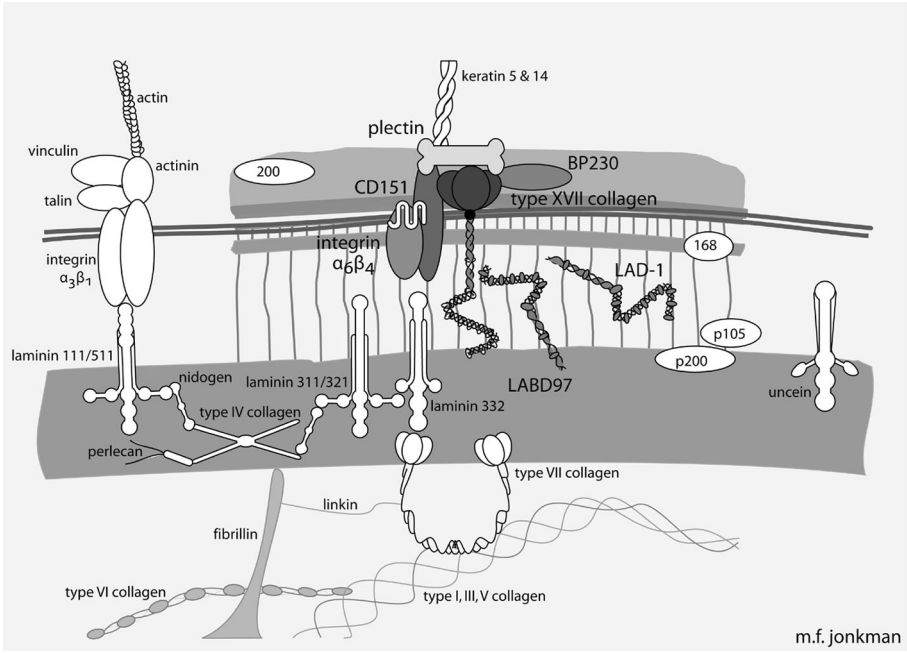

Figure 2. Schematic depiction of adhesion molecules at the basement membrane zone and hemidesmosome.
Drawing by prof. M.F. Jonkman.

Yeast-two-hybrid studies revealed several putative interactions between type XVII collagen and the other hemidesmosomal proteins.

Type XVII collagen interacts with BP230 through a region of 85 amino acids (145-230) in the N-terminal region and this sequence is both necessary and sufficient to incorporate BP230 into the hemidesmosome. BP230 is involved in this interaction by the NH_2-terminal Z-Y domain and to further strengthen the interaction additional binding sequences on type XVII collagen might be needed. BP230 itself then binds the specific basal cell intermediate filament keratins 5 and 14 by the B and C domains of the COOH-terminus[48,49].
The amino-terminal first 400 amino acids of type XVII collagen are needed for binding to the Z-Y domains of plectin. Surprisingly, in the same assays, binding of type XVII collagen to a component of the desmosome, desmoplakin, was observed[49].
Integrin $\alpha_6\beta_4$ is also bound by type XVII collagen. The β_4-subunit binds through half of its connecting segment and its FNIII repeat, both located in the C-terminus, to type XVII collagen. Two distinct binding sites in type XVII collagen are involved in this interaction: one in the first 230 amino acids and a second more C-terminally located in the region encompassing amino acids 231-401[49-51]. The α_6-subunit binds to the NC16A domain, to be precise amino acids 506-519, of type XVII collagen, since cells incubated with an antibody against this domain were unable to form hemidesmosomes. Noteworthy, which sequence of α_6-integrin is involved is unknown[52,53].
Binding of type XVII collagen to the most recent discovered component of the hemidesmosome, tetraspanin/CD151, has not been reported yet. So far, only interaction with integrin β_4 is seen, but considering the function of tetraspanin in the early formation of hemidesmosomes (pre-forms) actual interaction with tetraspanin must seriously be considered[44].
Type XVII collagen binding to non-hemidesmosomal proteins is also observed. Keratin 18 is bound by eleven amino acids (no. 15-25) in the N-terminal region of type XVII collagen. Almost the same region (amino acids 13-25) is responsible for the binding to P120-catenin, and binding to the isoforms 1-3 and not to 4 is observed. As type XVII collagen P120-catenin can be phosporylated, and this may alter their interaction and thus their signalling functions maybe modulated in this way. The C-terminus of type XVII collagen binds to laminin 5 and this interaction may be important for the organisation of laminin 5 in the basement membrane zone[34,54,55].

Type XVII collagen is not present in type II hemidesmosomes, as is BP230. These hemidesmosomes only consist of plectin, integrin α6β4, and tetraspanin and they are found in other tissues such as colon and mammary epithelium. Type II hemidesmosomes co-localise with the cytoskeleton and their organisation is probably controlled by actin[44-46,56]. The precise function of type II hemidesmosomes is still unknown. They may act as adhesion structures in cells that lack expression of type XVII collagen and BP230. In keratinocytes they are also thought to function as early complexes during the first rapid phase of wound healing. They would then

stabilise the epithelial-extracellular matrix interaction without disturbing the healing process[46,57].

Assembly of the hemidesmosome

Koster *et al* proposed a model for successive incorporation of the individual components into the emerging hemidesmosome[49]. They assume, that it starts with the interaction of integrin $\alpha_6\beta_4$ with plectin and tetraspanin/CD151, and also with the extracellular matrix protein laminin 5, which is the major and the most important lamina lucida component[40].
This is followed by incorporation of type XVII collagen into the complex. The binding involves interaction with the already present plectin, since in cultured keratinocytes that expressed a mutant form of the integrin β_4 incapable to interact with plectin, only hemidesmosome-like structures were observed that had less adhesive power[58,59]. The binding of type XVII collagen with plectin only is not sufficient strong to induce assembly hemidesmosomes when integrin $\alpha_6\beta_4$ is absent.
Finally, BP230 binds to two components of the complex, integrin $\alpha_6\beta_4$ and type XVII collagen thereby stabilising mature hemidesmosomes.
In this model a crucial role for plectin in the incorporation of type XVII collagen in the hemidesmosome is assumed, since in the aforementioned β_4-mutant no co-localisation of mutated β_4 with type XVII collagen was observed. Plectin, therefore, seems necessary to activate the cytoplasmic domain of β_4-integrin for binding of type XVII collagen. Although BP230 and plectin are members of the same family, the plakins, BP230 cannot take over the ligand-role of plectin.
This critical role of plectin is, however, not fully supported by *in vivo* data as cells of plectin-deficient mice do actually form hemidesmosomes, although rudimentary and in reduced number. In these mice, reduced expression of other hemidesmosomal components is observed[60].
A possible explanation for the discrepancy between skin and cell culture may be the presence of an *in vivo* ligand for type XVII collagen, whereas such a ligand is absent in cultured cells. In the latter case *in vitro* assembly of hemidesmosomes would rely on plectin. In support of an unidentified ligand is the observation that keratinocytes of patients with integrin $\alpha_6\beta_4$-deficiency also contain hemidesmosomes consisting of all components except integrin $\alpha_6\beta_4$. Moreover, in β_4-deficient keratinocytes, cultured on collagen-coated coverslips, punctuated expression of type XVII collagen, resembling hemidesmosomal structures, was observed[61]. Such hemidesmosomes may have been assembled by the interaction of type XVII collagen with the unidentified ligand in the epidermal basement zone[49,61].

Signal transduction by hemidesmosomal adhesion molecules

As mentioned before binding of immunoglobulin to type XVII collagen releases interleukins 6 and 8 from keratinocytes. Also tissue-type plasminogen activator is released after binding of type XVII specific IgG antibodies and this release is in part due to a signal transduction event[35,62].
Furthermore, Kitajima *et al* showed that phosphorylation of type XVII collagen on serine residues and destruction of hemidesmosomes occurred simultaneously after TPA stimulation of DJM-1 cells. This collapse of hemidesmosomes is also observed after binding of either monoclonal antibodies against type XVII collagen or after addition of sera of patients with bullous pemphigoid to the cells[36,63].
Thus, type XVII collagen obviously is involved in signal transduction but new data have not yet emerged. Since most evidence regarding hemidesmosomal signal transduction has come from studies on integrin $\alpha_6\beta_4$ we will focus on these here. It seems that signal transduction is especially important in regulating the disassembly of hemidesmosomes. Recently, the presence of both type XVII collagen and integrin $\alpha_6\beta_4$ in lipid rafts – subdomains of the plasma membrane enriched in cholesterol and glycosphingolipids- was reported[64,65].
Various signalling proteins concentrate in these rafts and these are all palmitoylated. Similar integrin $\alpha_6\beta_4$ will only be present in these rafts if also palmitoylated. Whether type XVII collagen is also palmitoylated in these lipid rafts has not been investigated. Currently, it is only known that disturbance of lipid rafts leads to increased shedding, although actual proof of shedding is limited[64].
In contrast, assembly of hemidesmosomes does not require palmitoylated integrin $\alpha_6\beta_4$, and therefore signal transduction and adhesion functions for integrin $\alpha_6\beta_4$ appear to be independent events[65].
Specific serine residues of integrin $\alpha_6\beta_4$ can be phosphorylated in lipid rafts via the EGF-R, which activates both Fyn and Yes. These Src family kinases phosporylate in turn integrin $\alpha_6\beta_4$. It is hypothesised that phosphorylation of integrin $\alpha_6\beta_4$ initiates disassembly of hemidesmosomes, and reverse, dephosphorylation causes assembly[66].
Moreover, EGF-R activates PKC through PLC-γ and inhibition of PKC may prevent EGF-R mediated disruption of the hemidesmosome[67-69]. It is not known whether this process is reversible.
Future research to the role of type XVII collagen in the disassembly of hemidesmosomes must reveal whether type XVII collagen is of equal importance here as integrin $\alpha_6\beta_4$. The presence of type XVII collagen in lipid rafts and the ability of keratinocytes to phosporylate type XVII collagen are strong indications that type XVII collagen also might have a functional role in the disassembly of hemidesmosomes.
This is especially important in understanding squamous cell carcinomas (SCCs). Here we see reduced ability to form hemidesmosomes in combination with increased integrin $\alpha_6\beta_4$ phosphorylation[66]. Whether type XVII collagen can act in the same way is currently unknown. In SCCs, however, we see disturbed expression of type XVII collagen and this expression varies with the stage of the tumor[70].

Hemidesmosomes have long been seen as stable structures, in line with their anchoring function. Tsuruta *et al* demonstrated that they are in fact very dynamic structures. The time needed for assembly was shown by the use of GFP-tagged β_4 integrin and type XVII collagen. Fluorescence after photobleaching was recovered by five minutes for β_4 integrin and somewhat longer for type XVII collagen. Due to this fast turnover, cells can rapidly re-organise their hemidesmosomes when necessary as in wound healing and division[71]. This dynamic behaviour fits in the proposed complex regulation.

Tissue distribution of type XVII collagen

Strong type XVII collagen expression is observed in various tissues with a prominent epithelial component other than skin. Multiple tissue RNA blots revealed expression in placenta, trachea, salivary and thyroid glands, colon, mammary, and prostate. No *COL17A1* mRNA expression was, however, observed in lung and kidney tissue, in which also epithelial cells are present. In contrast, tissue staining does reveal type XVII collagen in human bronchial epithelium[54,72].
Breast epithelia express type XVII collagen in the basal cells of the normal ducts and in glandular and surface epithelia of the endometrium mostly weakly intracytoplasmic and cell membrane-associated type XVII collagen expression was observed. The staining pattern was more diffusely distributed compared to expression in basal keratinocytes of the skin[73,74].
Surprisingly in mouse, *COL17A1* mRNA expression was observed in heart tissue in as well embryonic, neonate as adult stages. Expression of *COL17A1* mRNA was higher in adult and embryonic tissue[75]. So far, these findings have not been confirmed but it is very interesting considering the association of bullous pemphigoid (in which type XVII collagen is the key target antigen) with acute myocarditis[76]. However, we examined human heart tissue with monoclonal antibodies to type XVII collagen and found no binding (unpublished data).
Recently, Claudepierre *et al* found type XVII collagen expression in the retina, cerebellum, and the olfactory bulb. They suggest that type XVII collagen complexes with laminins in the central nervous system, although evidence for direct binding could not be established. This hypothetical complex may result in the stability or adhesion of synapses in the CNS[77]. Dissecting the functional role of type XVII collagen therefore does not only benefit our knowledge of cell biology of the skin but also of other organs.

Type XVII collagen and disease

Scientific interest became for the first time focused on type XVII collagen when it appeared to be an antigen in autoimmune bullous disorders. Today it is recognised as the most important antigen in the pemphigoid group of subepidermal bullous diseases. A further flurry of activity aroused when it was discovered that genetic deficiency of type XVII collagen results in the hereditary blistering disease non-Herlitz Junctional Epidermolysis Bullosa (nH-JEB). Additional interest comes from the fields of oncology and wound healing in which the hemidesmosomes are an important study subject considering its adhesive and signal-transduction functions.

Pemphigoid

The first disease in which type XVII collagen was found as one of the autoantigens was bullous pemphigoid (BP). Over the years it became clear that antibodies to the same protein also evoked other forms of pemphigoid. Today the presence of anti-type XVII collagen autoantibodies has been confirmed in pemphigoid gestationis (PG), linear IgA disease (LAD), ocular cicatricial pemphigoid (OCP), lichen planus pemphigoides (LPP), and mucous membrane pemphigoid (MMP). The antibodies bind to type XVII collagen and deposit in skin and/or mucosa and lead to dermal-epidermal separation. In direct immunofluorescence on tissue sections the deposition can be visualised as a linear staining pattern along the basement membrane (BMZ) (Fig. 3). Since the autoantigen role of type XVII collagen is best studied in BP, we here will further focus on the role of type XVII collagen in BP only[78,79].

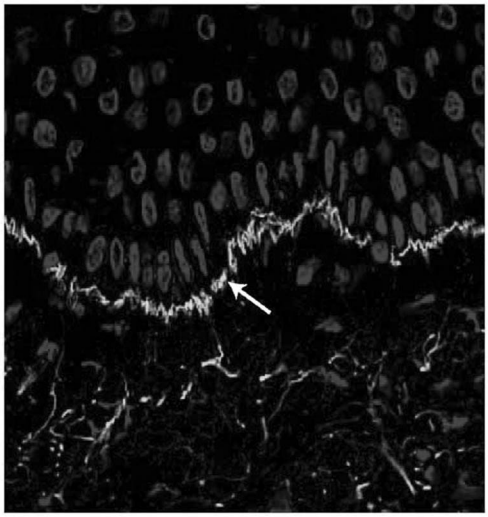

Figure 3. Autoantibodies bind to type XVII collagen and deposit along the BMZ in skin, leading to dermal-epidermal separation.
Direct immunofluorescence with antibodies against human IgG on skin tissue visualises the deposition as linear staining (arrow).

Evidence for the pathogenic role of the autoantibodies against type XVII collagen has been demonstrated both in *in vitro* and in *in vivo* studies. A fine example is the in vitro induction of separation of the epidermal and dermal compartment of skin by IgG autoantibodies from BP patient sera. Cryosections of healthy skin were first incubated with serum samples of BP patients, which was then followed by incubation with complement-containing serum and lymphocytes from healthy donors. This caused subepidermal separation and when the experiment was repeated with purified IgG from the same sera it became clear that this IgG was the initiating component of the separation process. When complement was omitted from the serum separation still occurred, when leukocytes were omitted no separation was observed anymore[80].

Moreover, injection of IgG antibodies against the murine homologue of type XVII collagen into neonatal mice caused all key features of BP. Histological examination showed broad subepidermal vesicle formation and near the edges of the blisters in this BP model large numbers of neutrophils were present. Immunological examination demonstrated that increasing the amount of injected antibodies resulted in higher titres, which, in turn, were associated with increased cutaneous disease activity[81]. The importance of both these studies was that they demonstrated that the autoantibodies were indeed pathogenic and not, as was also suggested, merely an epiphenomenon.

The precise mechanism of the subepidermal separation by the autoantibodies is largely unknown, although the BP mouse model suggests an important role for neutrophils in this process. In other autoimmune diseases the crucial role of neutrophils in immune-complex-induced inflammatory tissue destruction had already been established. In the pathogenesis of these diseases neutrophils are attracted by activated complement factors and upon engagement of their Fc receptors, proteases are released[82]. Therefore it was assumed that neutrophils are also the cause of tissue separation in the subepidermal autoimmune diseases. The presence of the neutrophil proteases 97-kDa gelatinase B and neutrophil elastase in the mouse BP is in line with this assumption.

Liu *et al* showed that mice deficient for these proteases did not form blisters when used in the BP model. Recruitment and function of the neutrophils in the skin of these mice was not impaired by the genetic deficiency of the proteases. Recently, it was also shown that in man these same proteases are also involved in blistering. Induction of dermal-epidermal separation by IgG autoantibodies of BP patients was abolished when elastase- and gelatinase B-specific inhibitors were used. Therefore, the authors stated that these proteases must be responsible for the blister forming in bullous pemphigoid, although the precise interplay between these proteases is still unclear and currently unknown proteases may additionally participate in the separation. Knowing the responsible proteases may open new approaches for patient treatment[83-85].

Man is not mouse. This is reflected in the cellular infiltrates that differ in cell type. In mouse neutrophils are attracted by the autoantibodies, whereas in human BP

eosinophils are observed as early infiltrates. This complicates a simple adoption of the mouse model data to explain blister formation in human. At this moment no causal link between the presence of eosinophils and subepidermal blistering in humans is established[81,83]. On the other hand, an *in vitro* experimental blister model demonstrated the functional activity of neutrophils in human BP[86]. Thus, it remains quite possible that the neutrophils, which act in a later phase of the pathogenesis, do have the same role in man as they have in mouse.
A lot of effort has been put in identification of the different autoantigenic epitopes on the type XVII collagen molecule. Current opinion is that two distinct important regions exist and both are located in the extracellular part of type XVII collagen. The first region is the NC16A domain that is situated just extracellular of the transmembrane domain. The second region is the carboxyl-terminal including the COL15 domain. By immuno-absorbing the NC16A-specific antibodies, it was shown that these indeed induce skin separation while autoantibodies against the COOH-terminus, used at a same titre, failed in attracting leukocytes and inducing separation. The NC16A autoantibodies can be further divided based on the subregion of the NC16A they recognise. Dividing the NC16A in 5 parts, NC16A-1 to NC16A-5, demonstrated that the NC16A-1 to -3 (aa 492-534) is the dominant target area. The NC16A-4 area was shown to be uniquely recognised by lichen planus pemphigoides sera[87]. As discussed before the soluble ectodomain is also a target for autoantibodies. Besides shared epitopes with the full-length molecule it also contains unique epitopes that may be neo-epitopes[14,25,80,88,89].

T-lymphocytes in both BP and LAD patients are specific to nearly the same amino acid sequences compared to the autoantibodies. NC16A responsive T-lymphocytes appeared CD4 positive and produced a mixed Th1/Th2 cytokine profile. CD4 positive T-lymphocytes are responsible for promoting antibody production by B lymphocytes[90,91].
Also the subclass of the autoantibodies determines the pathogenic pathways followed in inducing blisters. Most studies reported IgG4, a non-complement-fixing antibody, to be the predominant anti-type XVII collagen antibody with IgG1 as the second. Thus, already at this level different pathogenic routes may be followed. Above that also anti-type XVII collagen IgA and IgE antibodies are present. The IgA may be important as it attracts neutrophils. In MMP the presence of both IgA and IgG autoantibodies does mark a more serious and more chronic course of the disease than the presence of IgG autoantibodies alone[92]. Kromminga *et al* found in 88% of BP patients such IgA by immunoblotting of recombinantly produced protein[93]. In routine immunofluorescence analysis on esophagus and split-skin such high percentages are not seen, which suggest that in BP the IgA levels largely are very low. Furthermore, also anti-type XVII collagen IgE was reported and the serum levels of this IgE followed the severity of the disease[94]. IgE-coated mast cells were detected in perilesional skin of the BP patients. Moreover, type XVII collagen peptides were detected on these mast cells. In addition, histamine was released from

basophils of untreated BP patients after stimulation of these cells with a recombinant NC16A domain[95]. These findings underline the possibility that also IgE autoantibodies are involved in the pathogenesis of BP.

It may seem disappointing that after two decades of research the exact pathogenesis of BP is still obscure. On the other hand it has been demonstrated that autoimmune mediated inflammation is rather complicated and in fact may consist of several distinct processes occurring simultaneously. Final victory will lie in cutting this 'Gordian knot'.

Hemidesmosomal Epidermolysis Bullosa

Genetic deficiency of either laminin 5 or type XVII collagen causes non-Herlitz-Junctional Epidermolysis Bullosa (nH-JEB)[32,33]. The underlying defect is mutation of one of the following genes: *LAMA3*, *LAMB3*, *LAMC2* (coding for the alpha, beta and gamma chain of laminin 5) or *COL17A1*. The subtype nH-JEB with pyloric atresia that is caused by mutations in either integrin β_4 or integrin α_6 is beyond the scope of this chapter. JEB is characterised by the impaired function and reduction of hemidesmosomes. The clinical phenotype includes generalized skin blistering, dental anomalies, universal alopecia, and nail dystrophy[32,61,96-98].
The classification is subject of some debate, since some researcher proposed the term Hemidesmosomal Epidermolysis Bullosa (HEB) in stead of JEB[99,100]. In addition, Pasmooij *et al* were able to distinguish the clinical more severe, generalized atrophic benign epidermolysis bullosa (GABEB), from milder localized atrophic benign epidermolysis bullosa (LABEB) phenotypes by immunofluorescent antigen mapping (Pasmooij *et al*, submitted). We will use the term HEB troughout this thesis.

Of the reported mutations most are nonsense and some are missense mutations. Nonsense mutations in *COL17A1* mRNA are thought to result in nonsense mediated mRNA decay (NMD), although this assumption is so far only supported by the up-regulation of mRNAs of NMD factors in HEB keratinocytes[101]. No data are available on the actual amount of mRNA decay in these patients. A lot of effort has been put in understanding phenotype-genotype correlation, but currently no clear correlation between the distinct mutations and the severity of the disease has been established[98].
Some missense mutations are located in one of the collagenous domains of type XVII collagen and that may lead to decreased stability of type XVII collagen. To what extent this influences proper functioning of type XVII collagen is also not fully known[102-104].

Very mild forms of HEB can be observed when one of the PTCs is removed by outsplicing of the mutated exon. In three reported cases splicing was in-frame,

involving respectively exon 22, 30, and 33, so a somewhat smaller, but obviously partly functional protein was expressed[105-107]. It is likely that the outspliced exons here coded for less essential protein sequences as the opposite was also observed. Chavanas *et al* described a case where the mutated exon 32 was also removed by exon-splicing. This patient however had all the classical HEB symptoms. No expression of type XVII collagen was seen in this patient. Chavanas *et al* suggested that the absence was caused by destabilisation of type XVII collagen due to the loss of possible proline hydroxylation sites. Two of these hydroxylation sites were lost by the deletion itself and due to the deletion two proline residues move from position Y to position X in the basic G-X-Y repeat[108]. In the X-position the proline residues are not hydroxylated and hydroxylation is a critical feature in the triple-helix stabilisation[109.]

However, no correlation between the number of lost X-position prolines and the phenotype of the patients could be found. Therefore, Pasmooij *et al* suggest that the involved exons may be important for ligand binding or signal transduction. If so, exon 32 has a more important function in this binding than the other three lost exons[105].

The exon 33 skipping, found by Ruzzi *et al*, was also present at a very low level in normal keratinocytes, indicating that this skipping may represent a constitutive alternatively spliced transcript that may be become upregulated under appropriate conditions[107].

The patient that had the exon 22 outspliced showed some features of another type of EB, the simplex type. Intracellular epitopes of type XVII collagen were hardly detectable, whereas the extracellular epitopes were present. This indicated that in this patient the shed ectodomain was present in the lamina lucida of the basement membrane zone. Apparently, in comparison to the classic HEB phenotype in which all epitopes are absent, the weakest point of the basement membrane had shifted from the lamina lucida to the intracellular part of the hemidesmosome here[106].

Three HEB patients are reported that were revertant mosaics. In these patients germ-line mutations were corrected in some of the somatic cells resulting in the re-expression of type XVII collagen in these cells. When the clusters of repaired cells are large enough patches of healthy skin are seen[110-112]. Small patches of revertant cells may not be sufficient enough to restore proper adhesive function since Pasmooij *et al* also find minor clusters in non-healthy skin. In combination with the finding that two out of eleven Dutch patients were mosaics they concluded that reversion probably happens more often and may be overlooked.

The big question here is of course whether somatic reversion is a driven process. However, calculating the chances that such a revertant mutation occurs revealed that the mosaic patches in these patients could also have resulted from random mutagenesis. Their most intriguing observation was that different repair mechanisms existed in the same patient. In both patients a second-site mutation abolished the

mutation in one area, whereas in another area of either one of the two patients respectively a back-mutation and gene-conversion refrained the original mutation[112].

Revertant gene corrections are naturally occurring phenomena and understanding them may benefit developing medical gene therapy. Since skin is an easy accessible organ not only *in vivo* but also *ex vivo* gene therapy is an attractive option. Several attempts using different approaches to correct type XVII collagen deficiency have already been made on cultured keratinocytes.
Trans-splicing between a β-galoctosidase construct with a *COL17A1* sequence containing a nonsense mutation and a *COL17A1* exon-construct resulted in restoring the correct coding sequence[113]. Transfection of these constructs into keratinocytes showed β-galactosidase expression in a low percentage of the cells, indicating that in these cells the nonsense mutation had been successfully replaced. Whether this approach is conceivable and efficient enough to restore endogenous *COL17A1* mRNA remains to be elucidated.
Seitz *et al* transduced HEB keratinocytes with a retroviral expression vector and also demonstrated type XVII collagen re-expression. When these cells were used to regenerate human skin on immune-deficient mice no clear clinical evidence of blister forming was seen. However, on the histological level subepidermal bulla formation was observed and the gene transfer approach in this study may not be durable enough for actual use in human[114].
In addition, many other aspects of gene therapy still have to be resolved before it can be successfully applied.
For effective gene therapy it will be necessary to transduce the epidermal stem cells, but at this moment still no effective label for identification of these cells is available. In *ex vivo* gene therapy replacing mutated skin with genetically engineered skin will need complete removal of the mutated stem cells at the targeted location in order to avoid competition with the repaired stem cells. Stem cells have been reported in the interfollicular epidermis, in the bulge region of the hair follicle, and in sebaceous glands, but it is still unclear what their respective function is in regeneration of the skin and its appendages[115].
Recently, it was reported that the bulge stem cells do not contribute to the epidermis in the absence of trauma, though[116]. After wounding, however, they rapidly respond by producing transient amplifying cells that populate the wounded area, but these disappear again after a few weeks[117,118]. This phenomenon may interfere with proper adhesion of the repaired graft and experimental graft studies may have to be performed that address this question. Due to their deeper location stem cells of the hair bulge will be more difficult to remove as those of the epidermis. Moreover, since surgical procedures to place *ex vivo* produced skin sheets may cause scarring and delayed wound healing other techniques must be sought[98].

Type XVII collagen in carcinogenesis

Several studies have addressed type XVII collagen behaviour in squamous carcinoma cell (SCC) carcinogenesis. When carcinoma cells become malignant and turn invasive they have to become detached from the basement membrane, so they need to lose their anchors.
Squamous carcinoma cells continue to express type XVII collagen in an aberrant manner expression (Fig. 4)[15,70,119]. Parika *et al* demonstrated that for oral SCCs the most intense staining was observed in higher grade tumours. She also demonstrated downregulation of collagen XVII in basal cells in mild dysplasias. This may reflect disturbed keratinocyte adhesion to the basement membrane.

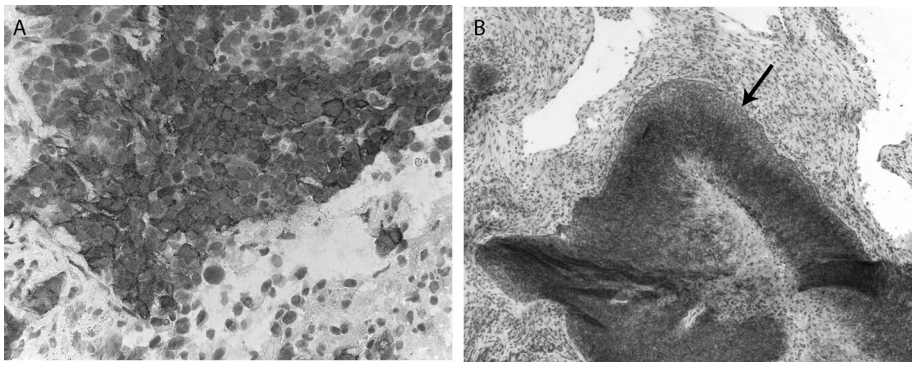

Figure 4. Aberrant type XVII collagen expression in squamous cell carcinoma.
Immunohistochemistry on moderate differentiated planocellular squamous cell carcinoma, using monoclonal antibodies against two different epitopes of type XVII collagen, shows strong expression in tumour nests (**A**) Cytoplasmic expression observed with 1A8C, an antibody against an intracellular epitope (**B**) An antibody against the extracellulair epitope 1D1 demonstrates, next to expression on the basement membrane (arrow), also similar strong expression in tumour cells (magnification: A: 400x, B 100x; H.H. Pas and H. van Goor, unpublished results).

Also ameloblastomas and basal cell carcinomas (BCC) demonstrate aberrant expression. In ameloblastomas, apart from expression in basal and suprabasal cells of the tumour nests, a diffuse intracellular staining was sometimes detected in the central cells of the neoplastic islands. In BCCs a similar diffuse cytoplasmic staining was observed in some central and peripheral cells of the tumour islands[120].
Bahadoran *et al* used an antibody to the soluble shedded type XVII collagen molecule and found faint or absent staining in tumours[121]. This might parallel the more cytoplasmic staining of the full-length molecule as seen by Parikka *et al* and could reflect an absence of shedding. Unfortunately no double staining with an antibody to the full-length molecule was performed.

In line, in another study of BCCs downregulation of *COL17A1* mRNA was shown as demonstrated by semi-quantitative RT-PCR. Downregulation of *COL17A1* mRNA was accompanied by a similar decrease in mRNA coding for other basement membrane components as BP230, integrin $\alpha_6\beta_4$, and laminin $\beta3$[120,122].
Also in neoplastic glandular epithelium of endometrial adenocarcinomas, type XVII collagen is expressed in variable degrees with increased synthesis observed in lower grade adenocarcinomas. As in other carcinoma types, cytoplasmic staining was observed, but here this was also seen in normal endometrium. Remarkable intensified expression was observed in foci of dispersed epithelial cells, and the authors wondered if this increased synthesis was a repair effort on enhanced matrix degradation due to metalloproteinases[74].
All above data demonstrate that type XVII collagen is actively regulated during the development of neoplasia, and during the invasive phase and the maturation of the tumour cells. Understanding why type XVII collagen is downregulated in mild dysplasia but upregulated in later phases as in SCCs will lead to better understanding of tumour development.

Wound healing

When basal cells start migrating to cover wounded surfaces they lose their hemidesmosomes, but after wound closure hemidesmosomes rapidly reassemble[123]. The knowledge on the exact role of type XVII collagen in this process is limited. Studies revealed that in the basal cells of the leading edge type XVII collagen is cytoplasmic rather than cell membrane bound[123,124].
This contrasts with the expression of the other membrane-bound hemidesmosomal molecule, integrin $\alpha_6\beta_4$, that is still observed at the cell membrane, although more uniformly and not only basal. This suggests different roles for these molecules. The cytoplasmic localisation of type XVII collagen is a fascinating observation as this is also observed in distinct phases of developing carcinoma (see above). Why and by what mechanisms type XVII collagen remains cytoplasmic rather than become organised in hemidesmosomes is yet unaccounted[57,123].

Unknown aspects of type XVII collagen: transcription and translation

In 1967 Jordon *et al* showed for the first time that the epidermal basement membrane contained an autoantigen that led to a blistering disease[125]. Nineteen years later Labib *et al* demonstrated the existence of an autoantigen of 180 kDa at the BMZ[126]. Today, after again a same period, we have gathered an impressive amount of information on this protein. As has become apparent in the preceding pages, many aspects of type XVII collagen have been thoroughly investigated. Studies have

been performed to its role in healthy tissue and cells and even more to its role in the pathogenesis of a range of diseases. Although these studies have provided a wagonload of information about type XVII collagen, certain aspects still are not fully understood. In the following paragraphs we will point out these aspects and we will argue the importance for further research.

Full-length type XVII collagen protein is additionally processed into two soluble forms, possibly by shedding of the cell membrane[14,19,27]. However, the involved sheddases could not completely account for the total of soluble forms, so the search for additional mechanisms is required[22]. In addition, no function is known for these other type XVII collagen proteins. Suggested functions include that both molecules may act as ligand or as a receptor for a yet unidentified ligand in the basement membrane.

Whether the alternative splicing of the *COL17A* gene into the two different mRNA forms is somehow connected with the appearance of the two soluble ectodomain forms remains elusive, and no evidence exists to support this hypothesis. Due to the fact that this alternative splicing entails the 3'UTR sequence, the function of the *COL17A* mRNA splicing may concern regulation of translation, since 3'UTRs are known to be capable of influencing translation levels, transcript stability, and mRNA transcript localisation. For instance, *cis*-acting sequences in the 3'UTR confer instability to mRNA transcripts which can be overcome by binding of *trans*-acting protein factors and mRNA transcript localisation is an important regulation mechanism for a variety of reasons:

1 to produce a local high concentration of protein
2 to segregate specific RNAs to particular organelles or subcellular structures.
3 to initiate cell lineages by sequestering localized mRNAs within a specific blastomere.
4 to produce a gradient of morphogen[127].

The latter two are especially important in embryonic development.
A further lack is the sequence of the 5'UTR. UTRs have been accepted as a major players in the complex process of initiation of protein synthesis[128]. Considering the proved involvement of both the untranslated ends of mRNA in numerous important mechanisms related with protein expression, we will focus on characterisation of both the 5'UTR and the 3'UTR of *COL17A1* mRNA in part of this thesis.

To achieve more information about the transcription and translation of type XVII collagen is of cardinal importance, seeing the participation and influence of type

XVII collagen in many processes in the epidermis, and other tissues as well. The deregulated expression of type XVII collagen in the pathogenesis of carcinomas and in wound healing is completely uncomprehended. Here, expression of type XVII collagen is not only decreased or increased, but also a shift from cell membrane to cytoplasmic expression is observed[15,57,70,119,123]. These changes may be regulated through 5'UTR or 3'UTR sequences of the *COL17A1* mRNA.
Moreover, also the involvement of type XVII collagen in the normal homeostasis of the keratinocytes needs attention. Its ligands are probably only partly known and we also do not know the function of the lateral pool of protein observed in the basal cells. It may be connected with sequence of incorporation of the hemidesmosomal components in emerging hemidesmosomes –as the subcellular location at which this occurs is still unknown- but it is also possible that it is connected in completely unknown interactions with neighbouring keratinocytes[49].
Circumstantial evidence in favour of type XVII collagen as signal transduction molecule can be found but hard data have not yet been presented[35]. Also the function of type XVII collagen in non-epithelial cells has barely been investigated[54].

Many studies have focused on the role of type XVII collagen in the pathogenesis of blistering diseases. They have confirmed the main function of type XVII collagen: the anchoring of the basal cell to underlying basement membrane. In HEB it is clear that absence of type XVII collagen leads to easy separation of dermis and epidermis. On the other hand it has also evolved that lower than normal levels, or even mutated forms of the protein, may still confer certain stability to the skin. Answering questions, as what is the minimal needed transcript level and how important are the protein subdomains for adhesion, will be important to come to appropriate gene therapy. Therefore we will also address the *COL17A1* mRNA transcript levels in HEB patients, and compare these with the clinical phenotypes.

Aim of the thesis

In this thesis we attempt to lay a basic fundament for understanding the regulation of transcription and translation. We will address the possible function of the alternative splicing by further characterisation of the untranslated regions of the *COL17A1* transcripts. The two starting points on which we direct our research will therefore be the undiscovered sequence of the 5'UTR of *COL17A1* mRNA and the unexplored function of the alternative 3'UTR ends. Furthermore, we will study *COL17A1* mRNA transcript levels in type XVII collagen deficient HEB patients to better understand the relationship between transcript levels, protein expression, and functional adhesion.

Hence, in **chapter 2**, we will address the unknown sequence of the 5'UTR and demonstrate that it is of a rather complex nature. **Chapters 3 and 4** are dedicated to the functional meaning of the two alternative 3'UTRs. In the first of these two chapters we investigate the effect of the alternative spliced 3'UTRs on the translation respective transcript levels. In the other chapter, the subcellular localisation of both *COL17A* mRNA transcripts is determined and, moreover, the implication of this subcellular localization of type XVII collagen protein is examined. In **chapter 5**, the mRNA levels of both *COL17A1* transcripts are determined in keratinocytes of a panel of HEB patients. Also the influence of nonsense mediated mRNA decay on these transcript levels is investigated by blocking of the NMD machinery by cycloheximide. In **chapter 6**, the findings of these studies are summarised and we will discuss what the implications of our findings are for further study of type XVII collagen.